# STUDIES ON ISOLATED CELL COMPONENTS
# III. A CYTOLOGICAL STUDY OF THE EFFECTS OF HEPARIN ON ISOLATED NUCLEI[1]

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## INTRODUCTION

THE addition of heparin to isolated rat liver nuclei results in the release of desoxyribonucleic acid (DNA) or a DNA-protein complex as evidenced by viscosity changes and by chemical analysis (1, 2). These observations are of interest in view of the reported inhibition of cell division by heparin and heparin-like substances (3, 4) and the occurrence of hyperheparinemia in fatally irradiated animals (5). The following study was undertaken in an effort to gain further knowledge of the effect of heparin on the isolated nucleus through the application of stains to fresh preparations and by the use of the Feulgen reaction.

## METHODS

*Studies with unfixed nuclei.* Nuclei were isolated by differential centrifugation from fresh rat liver in a sucrose-phosphate buffer at pH 7.1 (6). Since it has not been possible to eliminate the last traces of cytoplasmic contaminants from nuclear preparations, it was important to be able to identify and compare material of both nuclear and cytoplasmic origin in all preparations. Crude nuclear preparations were therefore used in order that cytoplasmic material would be regularly present and readily identified in both control and experimental preparations.

The fresh material was examined in the unstained condition or with methylene blue or methyl green, or with a modification of the Pappenheim pyronin-methyl green mixture. The latter was found useful for routine observations while making preparations.[3]

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[3] A satisfactory mixture for vital staining consists of 0.17 g methyl green, 0.06 g pyronin, and 100 ml of 95 per cent ethyl alcohol. One drop is dried on each slide.

The effect of heparin and of basic dyes on isolated nuclei was studied by inducing solutions containing these substances to flow past the nuclei under the cover slip. All heparin preparations used had an anticoagulant activity of 100 Toronto units per milligram and were commercial dry beef lung preparations. The dyes were products of National Aniline Division of the Allied Chemical & Dye Corp. and were certified by the Biological Stain Commission.

All studies with fresh nuclei were made at pH 7.1 in the salt-sucrose solutions used for nuclear isolation (6).

*Studies with the Feulgen reaction.* Fresh rat liver nuclei (6) were fixed, washed, stained, and cleared in 15 ml centrifuge tubes. Routine staining procedures were used throughout. After each step the nuclei were sedimented centrifugally, the supernate discarded and replaced by the next solution. After dehydration through absolute alcohol the stained material was mounted on slides in Euparal or Diaphane.

To study the effects of heparin 0.8 ml of loosely packed nuclei and cells was made up to 2 ml with Solution III (6) and 6 mg of heparin in 1 ml of Solution 1 was added. After 8 minutes, when the characteristic viscous material had developed, it was either fixed for 15 minutes or hydrolyzed without prior fixation. The material was then sedimented, washed, stained, and mounted.

Several fixatives were employed. Acetic-alcohol was unsatisfactory in that it produced clumps of material which when hardened by dehydration made impossible the preparation of thin flat mounts. 10 per cent formalin (pH 3.4) was rather surprising in its action. When added to a suspension of cells or nuclei the suspension became somewhat viscous. It could, however, be sedimented in the clinical centrifuge. The prolonged washing necessary to remove the formaldehyde resulted in an appreciable loss of material. Good staining preparations were obtained from the residue. Formalin completely failed to coagulate or precipitate the viscous substance produced by the addition of heparin to the suspension of nuclei or cells, consequently no slides could be obtained by this method. Allen's B15 fixative produced satisfactory coagulation and resulted in a precipitate fine enough to permit the preparation of slides of sufficient thinness for study under oil immersion. Prolonged washing in 70 per cent alcohol with the addition of lithium carbonate to each wash was necessary to remove the color due to the fixative. In some cases nuclei were stained by routine Feulgen methods without prior fixation.

Coleman's modification of the Feulgen reagent was used and resulted in a colorless solution of excellent staining quality. Optimum staining was obtained after 10 min. hydrolysis at 60° and one hour staining time. Allowing the stained material to stand too long (overnight) in alcohol resulted in a diffusion of color from the nuclei into the cytoplasm. Routine dehydration times avoided this diffusion.

## OBSERVATIONS

*The effect of heparin and basic dyes on fresh nuclei.* Fresh unfixed nuclei when treated with heparin were observed to fade until their outline was hardly visible. The separation of nuclei previously clumped gave evidence of extruded material sufficiently viscous to push the nuclei apart (2). Many

whole cells in the heparin treated preparations exhibited a clear perinuclear space seemingly free of granular material.

Nuclei treated with azure A (7) or zinc free methylene blue (dye concentrations 1:1000 in Solution I used for nuclear isolation) shrank rapidly, and in the presence of high concentrations of the dye stained very deeply. Methyl green in the same concentration did not stain the nuclei deeply and caused only slight shrinkage. No methyl green staining material comparable to the nuclear contents was seen outside the nuclei. The nuclear shrinkage with these dyes was very rapid indicating that the dyes penetrated easily.

Heparin reversed the effects of the dyes. The nuclei were observed to swell and fade rapidly. Methyl green and azure A staining material was observed around the nuclei. In nuclei very deeply stained with methylene blue the material extruded in response to heparin was often observed to be in the form of darkly stained rods forced out through openings on the surface of the nuclei (Fig. 6). The extruded rods exhibited a slight birefringence.

Similar shrinkage with azure A and subsequent swelling and release of stained material in response to heparin are shown in Figs. 4 and 5.

Figs. 1, 2, and 3 illustrate the same group of nuclei before treatment, after methylene blue (1:1000), and after heparin (1:500). The average volume of the methylene blue treated nucleus is about $\frac{1}{4}$ that of the original nucleus, while the same nucleus after dye and heparin treatment is approximately $\frac{5}{4}$ of the original size.

*Observations with the Feulgen stain.* As previously mentioned, the suspensions were deliberately designed to contain both nuclei and cells and do not represent the highest degree of purity which may be prepared by the nuclear isolation technique used (6).

The general picture of the normal (unheparinized) stained material is shown in Figs. 15–18. Most isolated nuclei are circular in outline with smooth margins. The internal picture varies slightly with the fixation employed.

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Fig. 1. Crude fresh rat liver nuclear preparation in salt-sucrose solution (Solution I).

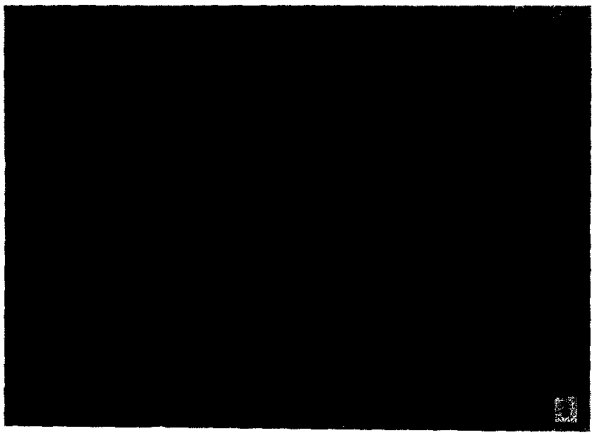
Fig. 2. Nuclei shown in Fig. 1 after treatment with zinc free methylene blue (1:1000) in solution I. Nuclei have shrunken to approximately $\frac{1}{4}$ their original volume. Few surface deformations are evident.

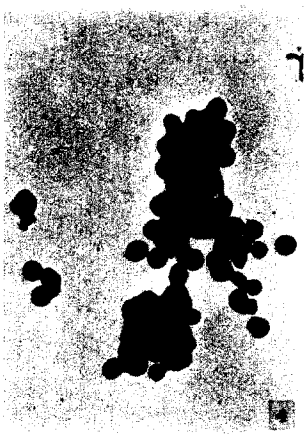
Fig. 3. Same nuclei as shown in Figs. 1 and 2 after treatment with heparin in solution I. Note extruded stained material on surface of nuclei. Much of the stain previously in the nuclei is now diffusely spread.

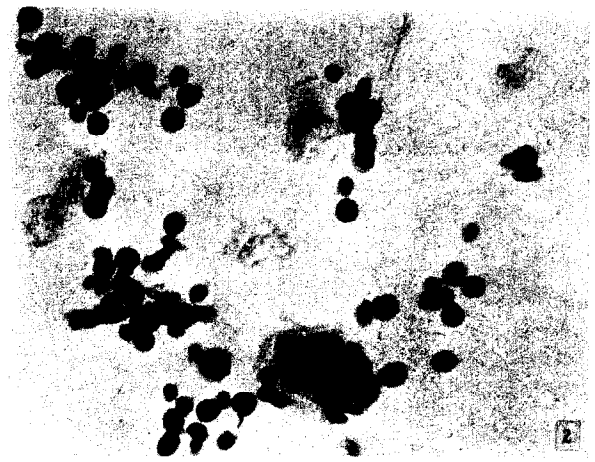
Fig. 4. Preparation of fresh rat liver nuclei after treatment with azure A (1:1000) in solution I. Little evidence of wrinkling of nuclear surface can be seen.

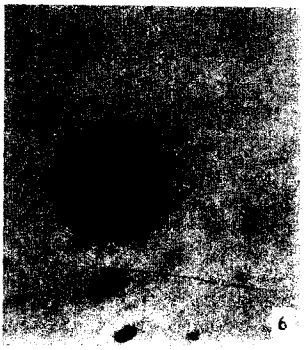
Fig. 5. Same nuclei as shown in Fig. 4 after treatment with heparin (1:500) in solution I. Azure A stained material could be observed to leave the nuclei.

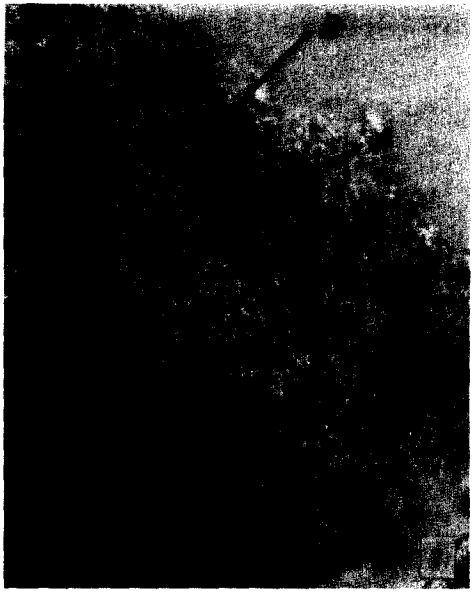
Fig. 6. Isolated nucleus treated with heparin after maximal shrinkage with methylene blue. Note rods of extruded stained material.

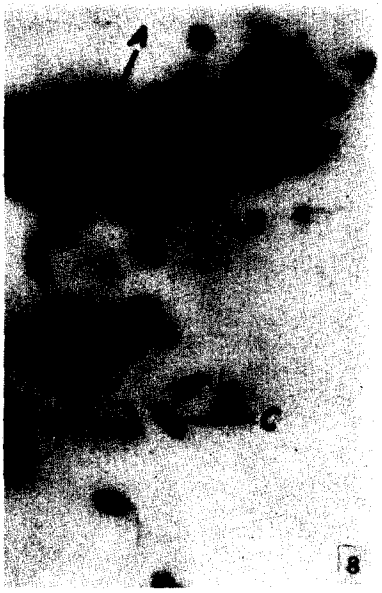










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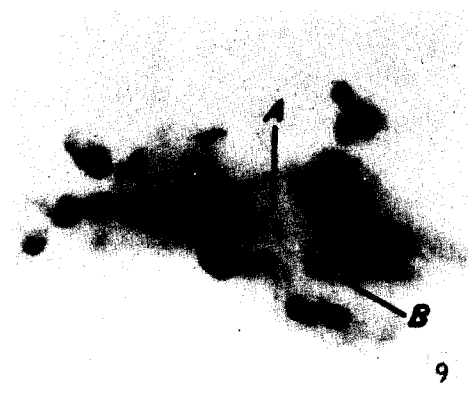


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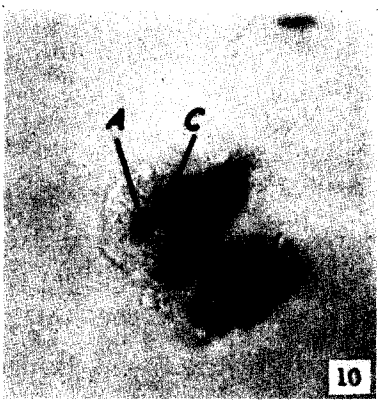


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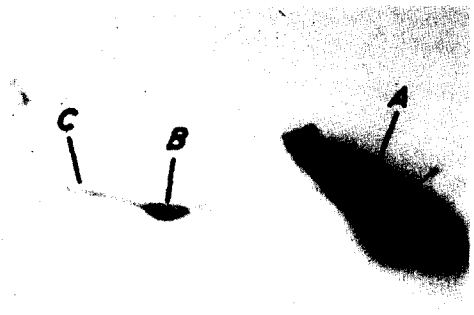


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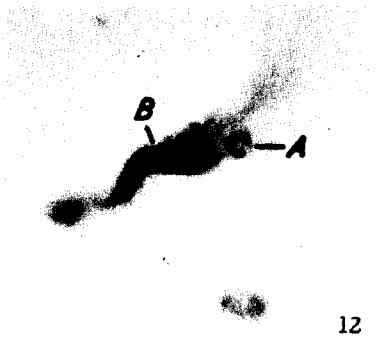


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The Feulgen positive material is slightly more granular after B15 fixation than after formalin, but is uniformly distributed in both. Nucleoli show as faint outlines, lighter in color and presumably Feulgen negative, but this could not be positively ascertained in whole nuclei. In the cells present the cytoplasm appeared finely granular and totally devoid of stain. The nuclei of these cells were similar to the isolated nuclei.

About one nucleus in five showed some irregularity in outline with evidence of damage to or rupture of the nuclear membrane. Usually this damage was slight but in some cases nuclei were partially disintegrated and sometimes pulled out into thick threads (Fig. 15). A small amount of Feulgen positive debris, apparently representing nuclear fragments, was present (Fig. 18). Feulgen negative coagulum was present in fairly large quantity and recognizable denucleated cells were frequently seen. A thin film of cytoplasm was adherent to many isolated nuclei. Internal vacuolization of nuclei was rarely observed.

White blood cells were a constant feature of these preparations (Fig. 16). Approximately one nucleus out of each hundred was that of a w.b.c. Although some w.b.c. showed evidence of damage most appeared normal, with no evidence of structural damage and with visually intact cell membranes.

Heparin treated nuclei stained well and exhibited varying degrees of change attributable to heparin. Practically all nuclei showed changes, marked irregularity in outline, shape distortions, and varying degrees of internal vacuolization (Figs. 13–14). Surprisingly, in view of the large quantity of viscous material shown to contain DNA by chemical analysis (2), there was only slight evidence of the presence of Feulgen positive material

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Fig. 7. Heparin treated, no fixation, Feulgen stain. Characteristic nuclear damage may be seen at A and in other nuclei. The coagulum, B, is Feulgen positive. A whole cell, C, contains Feulgen positive material in the cytoplasm. Some nuclei however show little sign of damage.

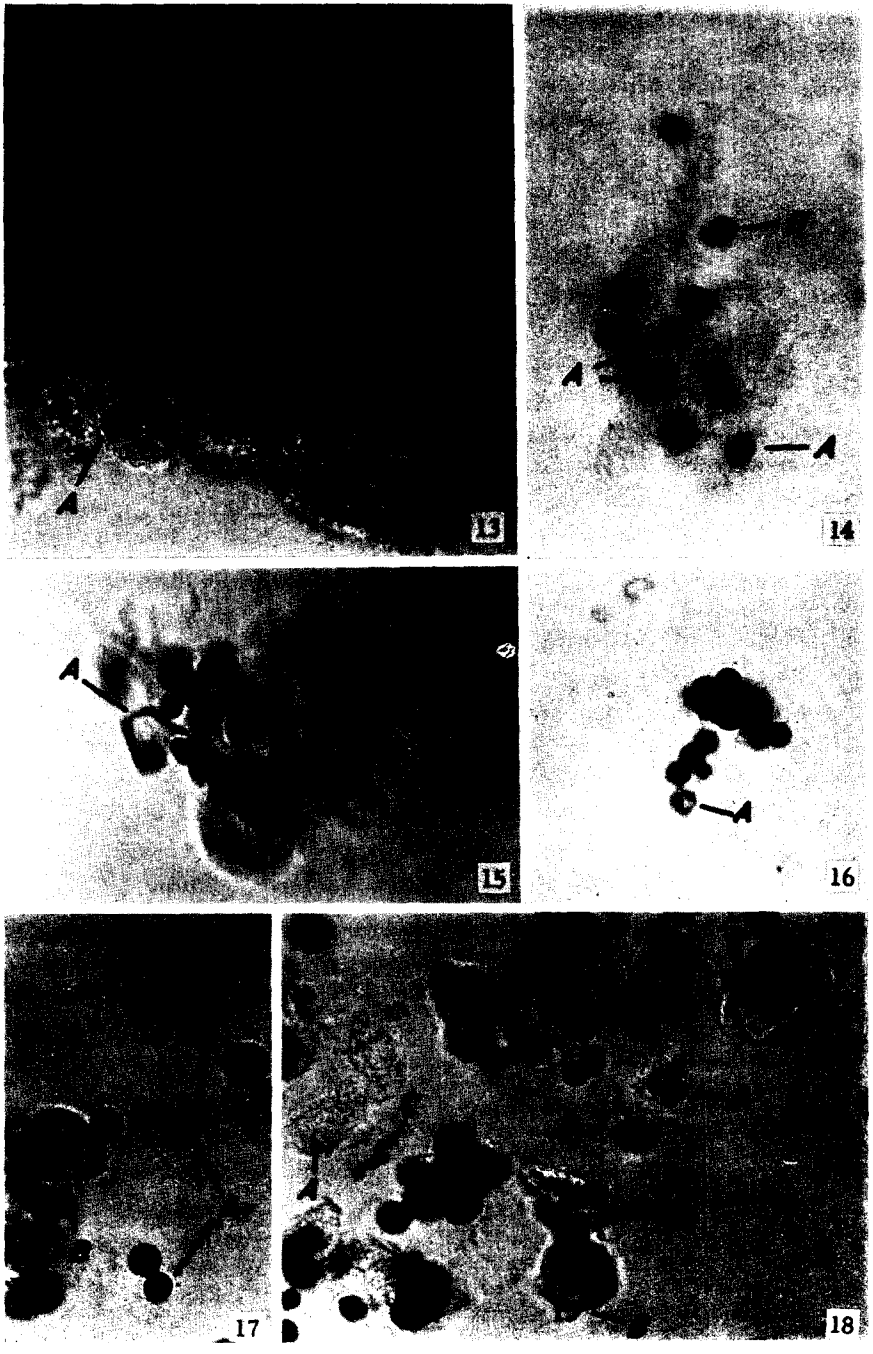
Fig. 8. Heparin treated, no fixation, Feulgen stain. A white blood cell showing no visible effects of heparin is seen at A. The coagulum, B, is Feulgen positive. The liver nucleus at C shows heparin damage, extreme vacuolization, and pale staining.

Fig. 9. Heparin treated, no fixation, Feulgen stain. Characteristic fibrous Feulgen positive coagulum is seen at A. An apparently undamaged white blood cell is seen at B.

Fig. 10. Heparin treated, no fixation, Feulgen stain. Whole cell A contains damaged nucleus. Feulgen positive material within the cytoplasm is present at C.

Fig. 11. Heparin treated, no fixation, Feulgen stain. Characteristic isolated liver cell nucleus after heparin treatment is seen at B with accompanying fibrous Feulgen positive material C. A dense mass of Feulgen positive coagulum with entrapped nuclei visible at A.

Fig. 12. Heparin treated, no fixation, Feulgen stain. Strongly Feulgen positive coagulum containing damaged nuclei at B, with adherent apparently undamaged white blood cell at A.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outside the nuclei. Large clumps of material gave a faint Feulgen test but this was seldom seen in isolated bits of tissue and coagulum. Three additional tests were performed with the quantity of heparin increased to 28 mg per 1.25 ml of packed nuclei and cells, and hydrolyzed between 6 and 20 min. In each case it was difficult to establish positively the presence of Feulgen positive material outside the nucleus.

In an effort to demonstrate more clearly the extra-nuclear DNA known to be present, resort was made to staining without prior fixation. Control (non-heparinized) nuclei were hydrolyzed without fixation and stained in the usual way. These slides gave the same general picture already described. It is noteworthy that a more intense stain was always obtained with the unfixed material than with any of the three fixatives tried. Heparinized nuclei were prepared as already described (0.8 ml nuclei and 6 mg of heparin) and hydrolyzed and stained without fixation. Figs. 7–12 show the effects of this treatment. Staining was intense and revealed the presence of large amounts of extranuclear Feulgen positive material in the form of fibers, strands, and thick masses. Nuclear damage was great, with marked vacuolization.

The nuclei of the white blood cells present did not differ in appearance from those seen in the control preparations. Damaged w.b.c. were seen in control preparations but were not seen in heparin treated material. Liver nuclei present in isolated whole cells and completely enclosed by cytoplasm were frequently as drastically affected as isolated nuclei (Fig. 13). Since the whole liver cells observed constantly showed irrregular margins, frequently with gross tears, the cell membranes cannot be presumed to be intact.

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Fig. 13. Heparin treated, Allen's B15 fixation, Feulgen stain. Heparin damaged nucleus within whole cell at A. Other damaged nuclei are seen at B. The nucleus within a whole cell at C appears normal. Cytoplasm and coagulum appear to be very faintly Feulgen positive.

Fig. 14. Heparin treated, Allen's B15 fixation, Feulgen stain. Characteristic nuclear damage seen at A. The white blood cell at B does not show heparin damage. The fibrous coagulum appears Feulgen negative.

Fig. 15. No heparin, formalin fixation, Feulger stain. Distorted nucleus A is pulled out into a Feulgen positive thread. Other nuclei when in focus appear normal.

Fig. 16. No heparin, no fixation, Feulgen stain. Normal nuclei are shown. A somewhat out of focus white blood cell is seen at A.

Fig. 17. No heparin, formalin fixation, Feulgen stain. Normal nuclei are seen at A and in other areas of the photograph. A whole cell is shown at B. The cytoplasm is colorless. Feulgen positive debris, presumably a nuclear fragment, is seen at C.

Fig. 18. No heparin, no fixation, Feulgen stain. Cytoplasmic debris, Feulgen negative, is seen at A. A whole cell showing damage to the cell membrane is seen at B. Feulgen positive debris appears at C. Most nuclei appear normal but occasional slight distortions may be seen at D. and elsewhere.

An interesting difference in the effect of the preliminary acid hydrolysis on unfixed heparinized and non-heparinized samples was noted. The supernate of the heparinized sample remained clear while the supernate of the non-heparin treated sample became cloudy. High speed centrifugation failed to sediment this cloudy supernatant material. The addition of heparin to the cloudy supernate resulted in the formation of a fine white easily sedimented precipitate which proved to be Feulgen negative. It could also be precipitated by ether-alcohol and gave the acid-mercuric chloride test for histone (11) and a positive reaction with a micro-biuret test (10). The supernate of the heparin-treated preparation which was also Feulgen negative failed to give a positive test for histone or protein and gave no precipitate on the addition of heparin.

## DISCUSSION

In attempting to confirm the presence of DNA in the heparin produced extra-nuclear gel, use was made of azure A, methyl green, and methylene blue staining. Positive tests were obtained with all three on the extruded gel. However it was noted that pretreatment of the nuclei with these stains (1 : 1000 in a sucrose-phosphate buffer) resulted in a sudden drastic shrinkage in the case of azure A and methylene blue, and a slight shrinkage with methyl green. These effects were reversed when heparin was introduced in the same buffer, the nuclei promptly swelling to more than their original size. Stained material could now be identified outside the faded nuclei. These results are taken as additional confirmation of the release of DNA by heparin from the isolated nucleus (6).

Nuclei maximally stained with methylene blue when treated with heparin were observed to extrude rods of stained material through apertures scattered over the nuclear surface. The birefringence observed in these rods suggests some degree of orientation of DNA molecules in the extruded material. It should be noted that the existence of regularly spaced apertures in the surface of the immature frogoocyte nucleus has been previously reported (8).

No wrinkling was evident in the rapidly shrinking nuclei until maximal shrinkage had been obtained, at which time slight irregularities were occasionally observed. The surface of nuclei isolated by the procedure used here (6) seems therefore to possess very elastic properties.

The rapid shrinkage in the presence of positively charged dyes and the rapid swelling in the presence of the negatively charged heparin molecules are characteristic of the behavior of negatively charged gels.

It has been pointed out previously (6, 2) and should be emphasized again that there is no way of determining at this time what changes may have

occurred in nuclear ultrastructure during the isolation procedure. No definite conclusions can therefore be made as to whether the observations on isolated nuclei are directly applicable to intact intracellular nuclei.

As further evidence of the nucleic acid releasing action of heparin on isolated liver nuclei, the presence of Feulgen positive material outside the nuclei has been shown. No final conclusions can be drawn from these observations but they invite and provide some basis for speculation as to the mode of action of heparin on cell components.

It has been noted that although isolated liver nuclei and those within liver cells show characteristic heparin damage, the nuclei of white blood cells whose cell membranes were visually intact showed no such damage. There seems to be no reason to assume a chemical difference in leucocyte nuclei to account for this resistance. Liver nuclei completely enclosed in cytoplasm were not protected, but in these cells the cell membrane was clearly not intact. This suggests that with the heparin concentration used either the intact cell membrane is not permeable to heparin or that more nearly normal cells (leucocytes) are not as subject to heparin damage as are injured cells.

Acid hydrolysis of fresh tissues has been used to free them of histone (9) and such hydrolysis was necessary in these studies to demonstrate the presence of extranuclear Feulgen positive material after heparin treatment. When freshly prepared suspensions of nuclei which did not contain heparin were subjected to acid hydrolysis as in routine Feulgen procedure, a cloudy non-sedimentable supernate resulted which was precipitated by heparin and gave positive protein (biuret) and histone (acid-mercuric chloride) tests. The supernate of hydrolyzed heparin treated suspensions remained clear, formed no precipitate with heparin and failed to give positive protein and histone tests. This suggests that the action of heparin may be the replacement of DNA in the nucleohistone of the nucleus by heparin, resulting in the structural damage noted, the release of DNA from the nucleus, and its subsequent partial combination with other protein, to form the previously described (1, 2) viscous material.

It has not been possible to determine positively why fixed preparations failed to show extranuclear DNA. Washing is essential to remove the fixative, and washing without prior fixation introduces additional factors. Either fixing or washing or both may be responsible. However, the fact that DNA, displaced from the nuclei by heparin (a naturally occurring substance) fails to give a positive Feulgen test when the customary fixative are used, but does give a positive Feulgen reaction without fixation suggests that the test depends to some extent on the manner in which the DNA is bound. This suggests

the possibility that the Feulgen reaction as ordinarily used may indicate some part of the nuclear DNA which may or may not be a constant fraction of the total DNA.

## SUMMARY

The gel released from isolated rat liver nuclei in response to heparin treatment has been found to stain with methylene blue, azure A, and methyl green when the dyes were added to the salt-sucrose nuclear isolation medium.

Azure A and methylene blue caused rapid nuclear shrinkage to as little as $\frac{1}{4}$ the original nuclear volume. Subsequent treatment with heparin caused the nuclei to fade rapidly and swell to approximately $\frac{5}{4}$ of the original volume. With methylene blue stained nuclei heparin caused the extrusion of deeply stained, slightly birefringent rods through apertures on the nuclear surface. Methyl green also caused nuclear shrinkage, but to a lesser degree.

Studies with the Feulgen reaction demonstrated structural damage in isolated rat liver nuclei as a result of heparin action. The viscous material released by heparin was shown to be Feulgen positive by resort to hydrolysis without prior fixation, since after customary fixatives the presence of a Feulgen positive reaction outside the nucleus could not be clearly demonstrated. The possibility is suggested that the Feulgen reaction following the customary fixatives depends in part on the manner in which the DNA is bound.

The nuclei of leucocytes with visually intact cell membranes included in the nuclear preparations failed to show structural damage due to heparin and it is suggested that either the cell membrane provides some protection against heparin action or that damaged cells are more susceptible to this action.

Observations made provide additional basis for the conclusion that heparin replaces DNA in the nucleo-histone of the nucleus, resulting in the structural damage observed, and releasing DNA in the form of a soluble viscous protein containing complex.

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